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Stable *in vivo* expression of the cystic fibrosis transmembrane conductance regulator with an adeno-associated virus vector

TERENCE R. FLOTTE*†‡, SANDRA A. AFIONE†, CAROL CONRAD*, SHARON A. MCGRATH*, RIKKI SOLOW†, HIDEMI OKA*, PAMELA L. ZEITLIN*§, WILLIAM B. GUGGINO*§, AND BARRIE J. CARTER†¶

*Eudowood Division of Pediatric Respiratory Sciences and §Department of Physiology, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, MD 21205; †Laboratory of Molecular and Cellular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892; and ¶Targeted Genetics Corporation, 1100 Olive Way, Suite 100, Seattle, WA 98101

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ABSTRACT **Adeno-associated virus (AAV) vectors expressing the normal cystic fibrosis transmembrane conductance regulator (CFTR) cDNA complement the cystic fibrosis (CF) defect *in vitro*. Unlike other DNA virus vectors, AAV is a stably integrating virus, which could make possible long-term *in vivo* complementation of the CF defect in the airway epithelium. We report AAV-CFTR gene transfer and expression after infection of primary CF nasal polyp cells and after *in vivo* delivery of AAV-CFTR vector to one lobe of the rabbit lung through a fiberoptic bronchoscope. In the rabbit, vector DNA could be detected in the infected lobe up to 6 months after administration. A 26-amino acid polypeptide sequence unique to the recombinant AAV-CFTR protein was used to generate both oligonucleotide probes and a polyclonal antibody which allowed the unambiguous identification of vector RNA and CFTR protein expression. With these reagents, CFTR RNA and protein were detected in the airway epithelium of the infected lobe for up to 6 months after vector administration. AAV vectors do, therefore, efficiently promote *in vivo* gene transfer to the airway epithelium which is stable over several months. These findings indicate that AAV-CFTR vectors could potentially be very useful for gene therapy.**

Cystic fibrosis (CF) is a common single-gene disorder (1) characterized by a primary defect in cAMP-mediated stimulation of chloride secretion in the airway epithelium. The goal of CF gene therapy is to express CFTR in the airways to prevent the sequelae of this basic defect. *In vivo* expression of recombinant CFTR has been accomplished with adenovirus (2) and liposomes (3), and retrovirus vectors have proved efficacious in rat tracheal xenografts (4). Adeno-associated virus (AAV) vectors have some advantages over these vectors for CF gene therapy, because AAV is nonpathogenic (5) and takes advantage of a natural mechanism for high-frequency stable integration. Because AAV is an integrating virus there is potential for long-term correction of defective CF transmembrane conductance regulator (CFTR) function.

AAV was initially isolated as a tissue culture contaminant and was later found as a nonpathogenic coinfecting agent during an adenovirus outbreak in children (5). It is a single-stranded DNA virus of the parvovirus group, with a 4.7-kb genome. It requires coinfection with a helper virus, usually an adenovirus or herpesvirus, for efficient replication (6). In the absence of helper virus infection, AAV stably integrates at high frequency, often to a specific site on chromosome 19 (7–10). The integrating phase of the AAV life cycle can be exploited in the design of recombinant AAV vectors for gene transfer (11).

AAV vectors are capable of high-frequency integration in a CF bronchial epithelial cell line in culture (12), and AAV-CFTR vectors are capable of complementing the chloride transport defect in these cells (13). Here we describe successful *in vivo* gene transfer with an AAV vector. Stable AAV-CFTR vector gene transfer and expression were observed after selective bronchoscopic delivery to a single lobe of the New Zealand White rabbit lung or following transduction of primary CF nasal polyp cells.

METHODS

Plasmids and Vectors. The construction of the AAV-CFTR vector pSA306 has been described (13, 14). The AAV-CFTR plasmid pSA306 contains nucleotides 486–4629 of the CFTR cDNA sequence (15) flanked by the AAV inverted terminal repeats and at the 5′ end by a synthetic 60-bp oligonucleotide sequence. This vector produces a CFTR protein that is modified at its amino terminus to include a fusion peptide of 26 aa not found in native CFTR (MLLIYVHTKNQHTLIDASELFIRPGT) but fully complements the CFTR defect in a CF bronchial epithelial cell line (13, 14). Vectors were packaged into AAV transducing particles in adenovirus-infected 293 human embryonic kidney cells, concentrated by CsCl ultracentrifugation, and titered (12, 16).

Transduction of Primary CF Nasal Polyp Cells. Nasal polyp tissue from CF patients undergoing therapeutic polypectomy was dissociated by trypsinization. The primary cells were grown at 37°C with 5% CO_2 in LHC-8E medium in plastic tissue culture flasks coated with collagen, fibronectin, and bovine serum albumin (17). Cells (10^4) were seeded into microtiter wells. After the cells had attached overnight, they were infected with 10^7 particles of packaged SA306 vector (multiplicity of 1000 particles per cell). Cell cultures were then expanded by passaging weekly for 3 weeks for *in situ* PCR and immunofluorescence assays. Uninfected duplicate cell populations served as controls. Immunofluorescent staining was performed with polyclonal rabbit anti-CFTR antibodies (14).

***In Situ* PCR.** The *in situ* PCR amplification procedure (18, 19) was modified to include direct incorporation of digoxigenin-dUTP in the reaction mixture. Tissues which had been formalin-fixed and mounted on silane-coated glass microscope slides were predigested with the Viratype kit (Digene Diagnostics, Silver Spring) and after a "hot start" at 82°C for addition of primers and *Taq* polymerase, a 40-cycle PCR was performed with 6.0 mM Mg^{2+}; 200 μM digoxigenin-dUTP; 10 μM dTTP; 200 μM dATP, dCTP, and dGTP; 20 μM primers; and other reagents as described in the Perkin–Elmer/Cetus GeneAmp kit. Immunologic detection with the Boehringer Mannheim Genius 3 kit yielded a dark purple-brown product

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Abbreviations: AAV, adeno-associated virus; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; LUL, left upper lobe; RLL, right lower lobe.

‡To whom reprint requests should be addressed.

overlying cells which contained PCR products amplified from the vector DNA. Nuclear fast red was used as a counterstain.

Selective Bronchoscopic Delivery of AAV-CFTR Vectors. Ten 11- to 13-kg adult New Zealand White rabbits were anesthesized with intramuscular ketamine and underwent flexible fiberoptic bronchoscopy with an Olympus 3.5-mm pediatric bronchoscope. The orifices of the major airways were visualized and the tip of the scope was wedged in the right lower lobe (RLL) bronchus. In 8 of the 10 animals, 1 ml of vector stock containing 10^{10} SA306 (AAV-CFTR) vector particles dialyzed against Ringer's balanced salt solution, pH 7.4, was placed into the RLL bronchial lumen through the suction channel. In two animals which served as negative controls, 1 ml of Ringer's solution was placed in a similar manner. A non-instrumented animal served as an additional negative control. Four animals were sacrificed by pentobarbital overdose at 3 and 10 days postinfection (3 vector-treated and 1 vehicle-treated at each time point), and tissue samples were taken from both the targeted RLL and the left upper lobe (LUL). One additional animal was sacrificed at 3 months and another at 6 months post-instillation. Both proximal and distal bronchi and trachea were sampled. Formalin-fixed tissues were paraffin-embedded. Sections (5-μm) were used for the *in situ* PCR assay for vector DNA incorporation and for immunohistochemistry (20).

Immunodetection of CFTR Protein. A rabbit polyclonal serum antibody raised to a peptide on the amino-terminal side of the first nucleotide-binding fold of CFTR (181) was used as primary antibody in the nasal polyp studies (14), while two different chicken IgG antibodies were used in the rabbit experiments. The first was a chicken anti-human CFTR-R domain antibody, no. 602 (20). The second primary antibody, no. 934, was a chicken polyclonal antibody directed against the 26-aa polypeptide unique to the pSA306 vector (ML-LIYVHTKNQHTLIDASELFIRPGT). This sequence bears no homology to native CFTR and so was chosen to eliminate any potential difficulties due to crossreactivity with endogenous CFTR. The peptide was linked to thyroglobulin (20) and injected into female chickens to generate polyclonal antibodies.

Immunohistochemistry of rabbit tissue sections was performed with a 1:10 dilution of one of the two chicken primary antibodies and a 1:20 dilution of alkaline phosphatase-conjugated goat anti-chicken secondary antibody. Detection of second antibody was performed with the Kirkegaard & Perry Histo-mark Red detection kit. Immunoblotting of protein extracts of lung homogenates was performed as described (20). Aliquots (40 μg) of protein from the RLL of experimental and control rabbits were electrophoresed in an SDS/5% polyacrylamide gel, electroblotted onto nitrocellulose membranes, and probed with a 1:500 dilution of the primary chicken anti-fusion peptide antibody, no. 934. Secondary antibody reaction and detection were performed with the Amersham ECL chemiluminescence kit.

Reverse Transcription–PCR to Detect Recombinant CFTR RNA Expression. Samples (1 μg) of total cell RNA extracted (21) from lung tissue homogenates were treated with RNase-free DNase (Boehringer Mannheim) to remove any input vector DNA. Samples were then reverse transcribed with Moloney murine leukemia virus reverse transcriptase and the cDNA was then amplified in a 25-cycle PCR using primers spanning a region from exon 7 to exon 13 of the CFTR cDNA coding sequence. Duplicate samples treated as above without reverse transcriptase served as controls to confirm the adequacy of DNase treatment to remove vector DNA. The PCR products were then electrophoresed in a 1% agarose gel, blotted to nitrocellulose, and probed with a ^{32}P-end-labeled probe from CFTR exon 11.

RESULTS

Transduction of Primary CF Nasal Polyp Cells. To test the ability of AAV-CFTR vectors to infect primary human tissues, cells from nasal polyp tissue of CF patients were isolated and cultured, infected at passage 3 with the AAV-CFTR recombinant vector SA306 (13, 14) at 1000 particles per cell, grown for three more passages, and assayed for vector DNA transfer and CFTR protein expression. Over 75% of the cells (range, 76.2–90.3%; 275 cells counted) contained virus DNA. Likewise, >75% of the cells expressed recombinant CFTR protein (Fig. 1). Recombinant CFTR can be seen by immunofluorescence both in the plasma membrane and spread diffusely throughout the cytoplasm. Unin-

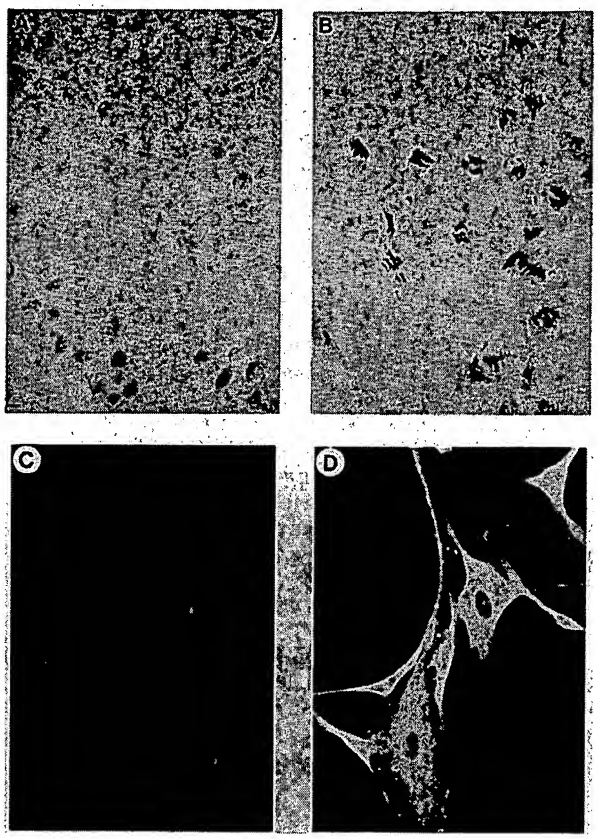

FIG. 1. Presence of vector DNA and recombinant CFTR protein in primary CF nasal polyp cells transduced with AAV-CFTR vector. Primary human CF nasal polyp cells were infected with the AAV-CFTR transducing vector (1000 particles per cell), grown for three more passages (3 weeks), and assayed for the presence of vector DNA incorporation by using an *in situ* PCR assay, with one primer chosen from a unique synthetic oligonucleotide sequence present in the 5′ end of the vector DNA and the other chosen from within the CFTR sequence. In this assay, predigestion of cells or tissues mounted on glass slides exposes nuclear DNA so that a PCR mixture can then be overlaid and used to amplify the vector-specific DNA sequences. We chose a nonradioactive marker, digoxigenin-dUTP, which is added to the reaction mixture and directly incorporated into any PCR product. An anti-digoxigenin–alkaline phosphatase conjugate was then used to localize the labeled PCR product in the cell samples. The presence of vector DNA is demonstrated (*B*) by the dark brown-black staining of the alkaline phosphatase-conjugated antibody bound to the digoxigenin-UTP nonradioactive label on the vector-specific PCR product. Uninfected control cell nuclei (*A*) are stained by the nuclear fast red counterstain. Recombinant CFTR protein expression from transduced cells is detected by strong immunofluorescent staining with a rabbit anti-CFTR antibody of the cytoplasm and membrane (*D*), whereas mock-infected cells show only faint background staining (*C*). (×200.)

fected control cells were entirely negative by *in situ* PCR and immunofluorescence.

Selective *in Vivo* DNA Transfer to Rabbit Airways. To determine the efficiency of *in vivo* gene transfer with the AAV-CFTR vector, the presence of AAV-CFTR vector DNA in rabbit lung sections was assessed by *in situ* PCR after selective delivery to the RLL through a fiberoptic bronchoscope. At either 3 or 10 days, vector DNA was detected readily in both the large (dark staining at arrows in Fig. 2*B*) and small airways and in the alveoli of the targeted lobe in each of the three vector-treated animals. None was found in the nontargeted control lobe, nor in any of the control animals (Fig. 2*A*) (nontargeted lobe, $n = 12$ sections; vehicle-treated, $n = 4$ sections; noninstrumented, $n = 2$ sections). The lobar bronchus from the proximal RLL consistently showed vector DNA incorporation in ≈50% of the nuclei (range, 51.2–60.8% of cells in a low-power field; 219 cells counted), including some nuclei near the basal lamina. In other cases, the entire cell appeared to contain vector signal. Distally, the pattern was more patchy. Some smaller airways showed vector DNA present in nearly 100% of the cells, whereas others had fewer or no positive nuclei.

In the rabbit sacrificed 3 months postadministration, vector DNA was still detectable in ≈50% of nuclei within the airway epithelium of the lobar bronchus of the RLL (Fig. 2*C*). By 6 months, however, the proportion of cells with detectable vector DNA signal had markedly decreased (Fig. 2*D*); ≈5% of nuclei from the epithelium of the lobar bronchus still gave a positive signal, mostly in small patches, as in the one shown in Fig. 2*D*. Very few positive cells were seen in the more distal portions of the lung at 6 months.

Several other tissues were screened for recombinant vector DNA. In addition to the uninfected LUL, the proximal trachea and kidney were examined in all six vector-treated animals by using the AAV-CFTR *in situ* PCR. Each of the kidney sections ($n = 12$) was negative, while only 4 of the 16 tracheal sections examined had some detectable positive nuclei (range, 5–25% in positive sections) at each of the time points examined. This was most likely due to coughing or mucociliary transport of vector particles at or shortly after the time of vector administration. Hematoxylin/eosin-stained sections of lungs, heart, liver, and kidneys were examined. There were no differences between controls and experimental animals, even as late as 6 months after vector administration. All of the treated animals remained clinically healthy throughout the experiments. These findings confirm that AAV-CFTR is not toxic either acutely or chronically.

Recombinant CFTR Protein Expression Parallels Vector DNA Distribution. The pattern of CFTR protein expression in the vector-treated animals was similar to that observed with vector DNA distribution. Both large and small airways and alveoli of the targeted lobe showed increased immunoreactivity with chicken polyclonal anti-human CFTR-R domain antibody 602 (pink staining in Fig. 3*D*) and anti-fusion peptide antibody 934 (pink staining in Fig. 3 *E–H*) 3 and 10 days after infection. Antibody 934 allowed the unambiguous distinction of vector-expressed protein from native CFTR, while antibody 602 directly confirmed that the CFTR polypeptide

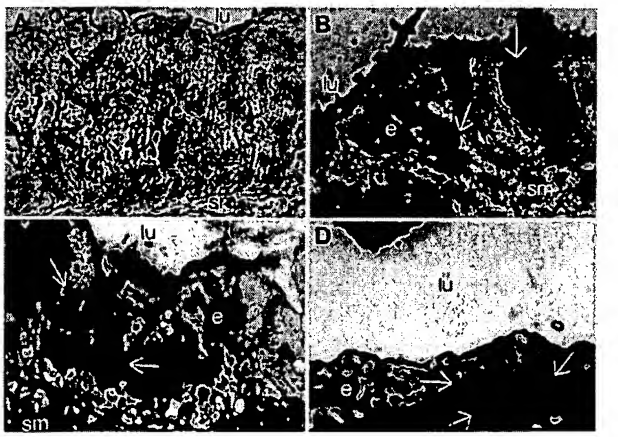

FIG. 2. Site-directed *in vivo* vector DNA transfer. Formalin-fixed 5-μm tissue sections were prepared from the lungs of rabbits sacrificed either 3 days or 10 days after targeted delivery of 10^{10} AAV-CFTR transducing particles or vehicle to the RLL. *In situ* PCR with vector-specific primers was used to probe for vector DNA. A nonradioactive digoxigenin-dUTP label was used and was detected with an anti-digoxigenin–alkaline phosphatase conjugate, which produced a dark purple-brown product over cells or nuclei containing vector DNA. Signal is seen in airway epithelial cells and more distal respiratory units at 3 days (*B*), 3 months (*C*), or 6 months (*D*) postinfection in the RLL, with the nontargeted LUL (*A*) for comparison. The number of positive cells decreased substantially by 6 months, but small patches (white arrows) of darkly staining cells were detectable. lu, Airway lumen; e, epithelial layer; sm, submucosal layer. (×220.)

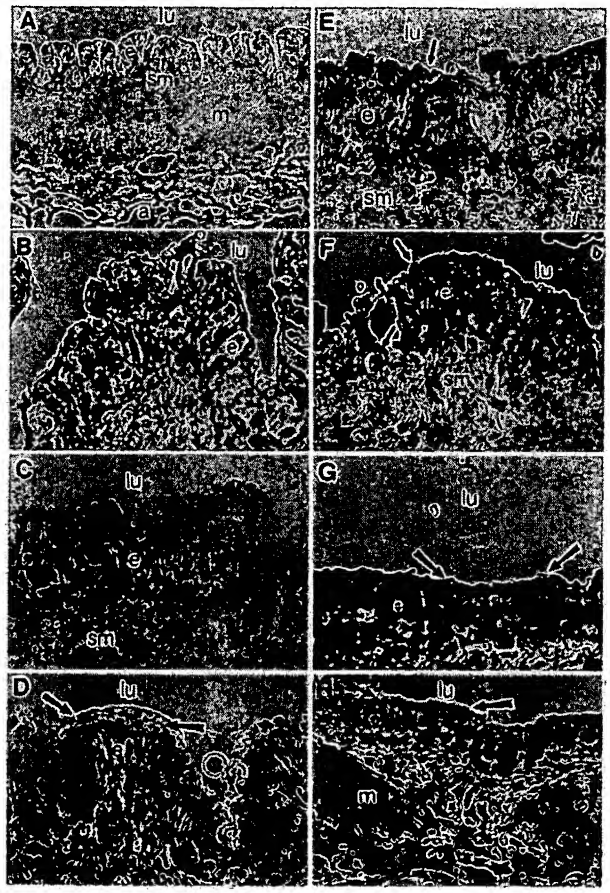

FIG. 3. Vector CFTR–fusion protein expression also persists in the airway epithelium up to 6 months after vector administration. The endogenous CFTR immunoreactivity with either chicken anti-R domain antibody 602 (*C*) or anti-fusion peptide antibody 934 (*A* and *B*) was consistently very low in the RLL of a vehicle-treated animal. By comparison, recombinant CFTR expression is seen along the airway surface in vector-treated RLL sections at 3, 10, 91, or 180 days postadministration by immunohistochemistry with antibody 934 (red staining at the arrows in *E–H*), or at 91 days with antibody 602 (*D*). Vector protein expression 3 months or 6 months after administration was less prominent and more patchy but was still clearly demonstrable with either of the antibodies. lu, Airway lumen; e, epithelial layer; sm, submucosal layer; m, smooth muscle. (×220.)

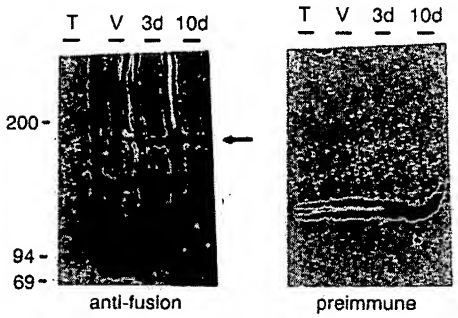

FIG. 4. Immunoblot to confirm specificity of chicken polyclonal antibody 934 for vector-expressed CFTR. Antibody 934, directed against the 26-aa fusion peptide sequence deduced from the nucleotide sequence of AAV-CFTR vector pSA306, was used to detect AAV-CFTR vector-specific protein expression. Protein extracts of samples harvested from vector-treated RLL at 3 or 10 days after vector treatment are shown (lanes 3d and 10d) along with a sample from an animal sacrificed 10 days after instillation of a bolus of Ringer's balanced salt solution (lane V), which serves as a vehicle control. A band migrating at ≈170 kDa (arrow) was observed with both of the vector-treated animals, but not in the vehicle control lane. A protein extract from the T84 colon carcinoma cell line (lane T), known to express large amounts of wild-type CFTR protein, served as an additional control to demonstrate the lack of immunoreactivity with native CFTR.

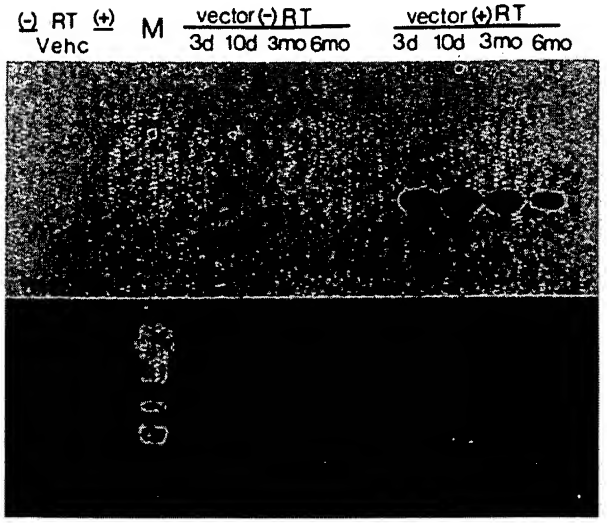

FIG. 5. AAV-CFTR RNA expression persists for 6 months after infection. Shown are both a Southern blot and an ethidium bromide-stained gel of products of a reverse transcription–PCR performed on DNase-treated samples of total cellular RNA extracted from lung homogenates harvested 3 days, 10 days, 3 months, and 6 months after vector administration. CFTR RNA expression at each of these time points in vector-treated animals is demonstrated by the amplified fragment. Control lanes include RNA from a lung homogenate from a vehicle-treated animal (Vehc) in which there is no signal. Duplicates of each sample handled as above but without reverse transcriptase [(−) RT lanes] demonstrate the completeness of vector DNA digestion.

sequence was expressed. Colocalization of CFTR immunoreactivity with antibodies 934 and 602 in serial sections provided additional evidence of the specificity of the immunodetection techniques.

In the large airways, the CFTR immunoreactivity was localized near the apical surface (pink staining in Fig. 3 *E–H*), in many cells analogous to that seen with endogenous CFTR. The controls, including the nontargeted LUL, the vehicle-instilled animal, and the non-instrumented animal, all showed very low levels of immunoreactivity with antibody 602 (Fig. 3*C*) or 934 (Fig. 3 *A* and *B*). CFTR protein expression persisted in patches of cells in vector-treated lobes at 3 and 6 months (Fig. 3 *D*, *G*, and *H*).

The specificity of chicken anti-R domain antibody 602 has been confirmed by immunoblotting (20). The specificity of anti-fusion peptide antibody 934 is demonstrated by the immunoblot in Fig. 4. Substantial amounts of a 170-kDa protein were seen in both of the samples from the vector-treated animals, but not in the sample from the vehicle-treated control animal. The T84 colon carcinoma cell line, which expresses large amounts of wild-type CFTR, did not demonstrate reactivity with this antibody, as expected, since this polypeptide does not contain any regions homologous to native CFTR.

Correlation of CFTR RNA and Protein Expression. To confirm that AAV-CFTR vector DNA was acting by increasing recombinant CFTR protein levels directly, and not by activating endogenous CFTR expression, we analyzed recombinant mRNA expression from tissue homogenates by reverse transcription–PCR. Signal was detectable at 3 or 10 days in vector-treated animals, but not in the vehicle-treated controls (Fig. 5). RNA transcribed from AAV-CFTR could still be detected 3 or 6 months after infection. The relative levels of mRNA expression could not be determined with the nonquantitative PCR assay.

DISCUSSION

The experimental models described here demonstrate the efficacy of AAV vectors for expression in primary human airway cells and for *in vivo* gene transfer. The presence and expression of vector genomes in cells from the airway surface epithelium were seen as early as 3 days after infection in the rabbits and persisted for at least 6 months. These findings indicate that the AAV vectors will be capable of long-term expression after *in vivo* gene transfer to the airway epithelium, an advantage for CF gene therapy.

The "epitope-tagging" of CFTR with a novel fusion peptide sequence allowed us to design vector-specific antibodies and probes. These reagents facilitated the distinction of vector RNA and protein expression from endogenous CFTR expression to establish biological efficacy in the current model. The current study also demonstrates the ability to physically target a gene therapy vector to a single lobe of the lung by fiberoptic bronchoscopy. The rabbit bronchoscopic model may provide a way to infect cells with the multiplicity of virus particles needed to infect a large percentage of cells in the airway, in the absence of a selective marker. An additional observation was that this targeted bronchoscopic delivery resulted in very little spillover to a nontargeted lobe, although there was some detectable vector DNA in the trachea of some animals. No vector DNA was detectable in any other organs, however, confirming the selectivity of vector gene transfer. Most notably, there were no pathological changes suggestive of inflammatory responses or neoplasia in the lungs or any other organs, suggesting that AAV-CFTR vectors are nontoxic and safe when delivered for airway gene therapy.

Several issues remain to be addressed. The long duration of expression observed *in vivo* may be due to AAV vector integration, which generally is quite stable *in vitro* (22). Since the lifespan of mammalian airway epithelial cells is probably <120 days (2), it is possible that the decrease in the numbers of cells with vector DNA and recombinant protein signal by 6 months was due to loss of the more terminally differentiated surface epithelium, with persistence of patches of cells derived from a stably transduced precursor cell population. The identity of precursor stem cells remains to be clearly under-

stood (4), however, and there is no direct evidence of integration in a self-sustaining stem-cell population.

These studies also provide insight into an important question regarding AAV vectors. It has been suggested that gene expression from AAV vectors, unlike that from retroviral vectors, may not require actively replicating cells (23). However, there has been little direct experimental evidence to address this hypothesis. Although the lifespan of airway epithelial cells is not well understood, it is clear that a large fraction of them are terminally differentiated and nonreplicating. Therefore, the efficiency with which the CFTR gene was expressed from the AAV vector *in vivo* provides evidence that gene expression from such AAV vectors probably does not require active cell division.

Other important questions remain regarding the safety and efficacy of AAV vectors for CF gene therapy in humans. The ultimate therapeutic efficacy of any CFTR gene vector depends on which cells will require CFTR expression for restoration of normal function of the intact airway and whether these cells will be transduced. It seems likely that these issues will not be resolved until clinical trials are performed. Likewise, safety questions regarding risks of inflammatory responses, complications of vector integration, and possible shedding of recombinant virus must be addressed in primate models and in human trials.

In summary, these observations show that a normal CFTR gene can be delivered in an AAV vector with high efficiency and result in stable expression in the relevant cell types both *in vitro* and *in vivo*. AAV-CFTR vectors may hold promise as a gene therapy for CF, since their potential for stable expression could make it possible to correct the basic pathophysiologic defect in the airway epithelium from an early age, prior to the onset of irreversible lung injury. If the relevant safety issues can be addressed satisfactorily, the use of such vectors in a human clinical trial will be warranted.

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